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Structure of an Acidic Exopolysaccharide of *Pseudomonas marginalis* HT041B

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The exopolysaccharide of *Pseudomonas marginalis* HT041B has been characterized as a 1,3-linked galactoglucan in which galactose and glucose are in the α- and β-anomeric configurations, respectively. The polysaccharide is substituted with pyruvate at the 4 and 6 positions of galactose and with succinic acid at either the 2 or 4 position of glucose. This polysaccharide has been given the trivial name marginalan.

In a recent report, we characterized the structure of the acidic exopolysaccharide (EPS) of the phytopathogen *Pseudomonas syringae* pv. *glycinea* as an alginate similar in structure to alginates isolated from *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. mendocina* (8). Subsequently, we examined the EPS of a wide range of plant pathogenic pseudomonads and found alginate production to be common among fluorescent types when glucose or gluconate was used as the carbon source in the culture medium; with sucrose as the carbon source, levan, levan and alginate, or alginate alone was produced, depending on the bacterial strain examined (3). On the basis of these results, we proposed that most, if not all, fluorescent pseudomonads are capable of synthesizing alginate. However, in recent experiments the EPS isolated from a strain of *P. marginalis* (an organism responsible for spoilage of fruits and vegetables in storage) grown on glycerol-containing medium was determined to be neither an alginate nor levan. As part of our investigation of bacterial EPS as a virulence factor of phytopathogenic bacteria, we undertook an examination of the structure of this polysaccharide.

General methods. Neutral sugar, uronic acid, and amino sugar analyses were done by colorimetric assays as previously described (9). Pyruvate concentration was determined by the method of Jeanes et al. (6), and succinate concentration was determined by the method of McComb and McCready (7). The method of permethylation analysis used in this laboratory has been described elsewhere (8). High-performance liquid chromatography (HPLC) analyses were carried out on an HP 1090 chromatograph (Hewlett-Packard Co.) fitted with a diode array detector and an HP 1037A refractive index detector, gas chromatography analyses were carried out on an HP 5880 chromatograph, and mass spectra were obtained on an HP 5990B GC-MS spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL 400X.

Preparation of EPS. *P. marginalis* HT041B was obtained from C. Liao (this laboratory). The bacterium was grown on *Pseudomonas* agar F (Difco Laboratories) for 2 to 4 days at 20°C. Mucoid growth was removed from the agar surface by using water and a bent glass rod. Cells were removed by centrifugation and subsequent filtration (0.45-μm filter). Crude EPS was dialyzed extensively against water at 4°C and was purified by the method of Sutherland (11). Protein content was further reduced to less than 1% by extraction with cold buffered phenol (5). The partially purified polysaccharide was then chromatographed on a column packed with DEAE-Sepharose CL-6B (Pharmacia, Inc.), using a 0 to 1 M NaCl gradient in 0.05 M Tris hydrochloride (pH 7.2) for elution. The fraction eluting between 0.5 and 0.6 M NaCl was collected.

Constituent characterization. Sugars were identified by gas chromatography analysis of the aldonitrile derivatives. Both pyruvate and succinate were identified by ^{13}C-NMR (Table 1) and HPLC and gas chromatography (methyl esters) co-chromatography with authentic samples.

Removal of substituents. Succinate ester was hydrolyzed at pH 11.5 (3 h, room temperature). The pyruvate ketal was hydrolyzed in 50 mM oxalic acid at 100°C for 90 min. In both reactions, the solutions were centrifuged, the supernatants were dialyzed, and the retained materials were lyophilized. Oxalic acid treatment also resulted in significant succinate hydrolysis.

Oligosaccharide preparation. EPS (50 mg) was hydrolyzed for 3 h at 95°C in 0.1 M H_2SO_4 (10 ml). After neutralization with $BaCO_3$ and deionization with Amberlite MB-3 resin (Sigma Chemical Co.), the sample was concentrated under a stream of N_2 to 1 ml. The sample was then fractionated by HPLC on an Aminex Q-15S Ca^{2+}-form column (2 by 30 cm; Bio-Rad Laboratories), using deionized H_2O as the mobile phase. The fractions corresponding in retention volumes to the tetra-, tri-, and disaccharides and glucose and galactose (as determined by maltoligosaccharide and monosaccharide standards) were collected, neutralized with $BaCO_3$, filtered, and lyophilized. A sample of the disaccharide fraction was hydrolyzed to monosaccharides (1 M H_2SO_4); as expected, glucose and galactose were the only sugars present.

Glycosidase assay. To an aqueous solution of a monosaccharide-free disaccharide fraction (1 mg/ml), 1 mg of glycosidase was added, and the solution was incubated at 37°C for 3 h. The solution was then boiled for 1 min to denature the enzyme, filtered (0.45-μm filter), and taken to dryness under a stream of N_2. The sample was divided into two fractions; one fraction was hydrolyzed with 1 M H_2SO_4, and both fractions were analyzed for monosaccharides in the usual manner.

NMR analysis. ^{13}C-NMR spectra were obtained in D_2O at ambient pH (ca. 5) and also, for desuccinylated samples, at pH 12. At the latter pH, line broadening was greatly reduced. About 10,000 scans were accumulated for each spectrum, using a 5-s pulse delay.

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TABLE 1. ^{13}C-NMR of *P. marginalis* HT041B EPS and chemically modified derivatives

Carbon	NMR (ppm)		
	HT041B	HT041B-S[a]	HT041B-(S+P)[b]
C-1 galactose	100.4	100.4	100.7
C-3 galactose	78.0	78.0	80.9
C-4 galactose	63.3	63.4	61.4
C-6 galactose	65.8	65.9	61.7
C-1 glucose	105.5	105.5	104.9
C-3 glucose	82.2	83.5	84.3
C-6 glucose	61.5	61.4	61.7
C-2 pyruvate	101.7	101.5	
CH_3 pyruvate	25.9	26.0	
COOH succinate	182.1		
COOR succinate	175.7		
CH_2 succinate	32.5		

[a] Succinate removed.
[b] Succinate and pyruvate removed.

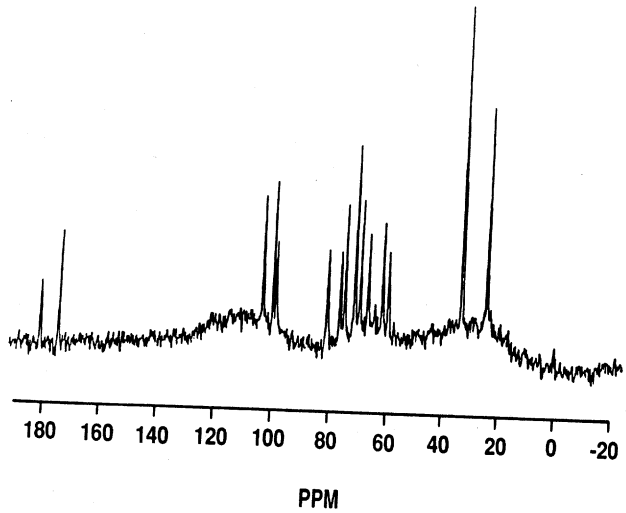
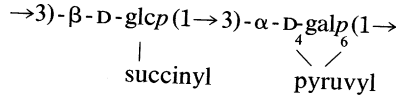


FIG. 1. ^{13}C-NMR spectrum of *P. marginalis* HT041B EPS. Shifts are given (in parts per million) downfield from sodium dimethylsilylpropionate (not shown).

The EPS of *P. marginalis* contained D-glucose, D-galactose, and the substituents succinic and pyruvic acid in an approximate molar ratio of 1:1:1:1. Permethylation analysis of the EPS indicated the presence of a 3-substituted glucose (identified as 2,4,6-trimethyl-1,3,5-triacetylglucitol) and a 3,4,6-substituted hexitol (identified as 2-methyl-1,3,4,5,6-pentaacetylgalactitol). Permethylation analysis of depyruvylated EPS indicated the presence of 3-substituted glucose (identified as described above) and 3-substituted galactose (identified as 2,4,6-trimethyl-1,3,5-triacetylgalactitol). Even though standards were not available to identify the penta-substituted alditol, the results for the pyruvylated and depyruvylated EPS permethylation analysis show unambiguously that the pyruvyl substituent is linked to galactose at carbons 4 and 6. The ^{13}C-NMR data (Table 1) were also consistent with this assignment. The NMR data indicated only two anomeric carbons, one α and one β. Attempts to degrade the polysaccharide with α- or β-glycosidase were unsuccessful. The polysaccharide was partially hydrolyzed to yield a mixture of oligosaccharides composed of predominantly disaccharides and lesser amounts of tri- and tetrasaccharides. The disaccharide fraction was isolated by preparative HPLC, and samples of this fraction (which we assume contain both possible disaccharides, which are not resolvable under the HLPC conditions used) were treated with α- and β-glucosidases and α- and β-galactosidases. Only treatment with β-glucosidase or α-galactosidase resulted in the release of glucose and galactose. These results, along with the permethylation analysis, indicate that the polysaccharide is a linear polymer with the repeating unit:



The NMR spectrum (Fig. 1 and Table 1) is consistent with these assignments (1). A large downfield shift of the C-3 of glucose, similar to that observed for laminarin (2), occurred at high pH. On the basis of the shift for the carbon of the incipient pyruvyl methyl group (25.9 ppm), the ketal carbon configuration is R (4). The position on glucose that is succinylated appears to be either C-2 or C-4 since there is no shift for C-6 upon desuccinylation, which is the only other possible position of substitution on the glucose moiety. At polysaccharide concentrations greater than 2 mg/ml, the viscosity of an aqueous solution was very high; therefore, it was not possible to perform 2-dimensional NMR experiments to determine the site of succinyl substitution. Attempts to prepare oligomers from the polysaccharide by treatment with available glycosidases and thus obtain solutions of lower viscosity were unsuccessful. We are now in the process of purifying an enzyme from *P. marginalis* that degrades its EPS which, hopefully, can be used to produce the desired oligomers for NMR analysis.

A similar EPS containing alternating β-1,3-linked pyruvylated galactose and acetylated glucose has been reported for *Achromobacter* species (12). This EPS was postulated to form complexes with heavy metals through the pyruvate carboxyl group as a means of detoxification (12). Also, Read and Costerton (10) recently reported the isolation of an EPS composed of glucose, galactose, and pyruvate (1:1:0.5 molar ratios) and variable amounts of acetate from single freshwater isolates of *P. putida* and *P. fluorescens*. It is now evident that alginic acid is not the sole acidic EPS produced by fluorescent pseudomonads.

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